

# Presence of an alien turtle accelerates hatching of common frog (*Rana temporaria*) tadpoles

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## Abstract

The presence of a predator affects prey populations either by direct predation or by modifying various parts of their life history. We investigated whether the hatching time, developmental stage, and body size at hatching of common frog (*Rana temporaria*) embryos would alter in the presence of a red-eared slider (*Trachemys scripta elegans*) as a predator. The presence of a predator affected all factors examined. We found that in the absence of the slider, the embryos hatched in 12 days, while hatching was accelerated by two days in slider treatment. At the same time, the embryos hatched smaller and at a lower stage of development with the slider than without it. Our study extends the range of predators studied, including the effect on different phases of development of potential amphibian prey.

## Keywords

Antipredator defence, embryonic development, invasive predator, predator-cued hatching, predation risk, *Rana temporaria*, *Trachemys scripta elegans*

## Introduction

The impacts of invasive species on native communities are still difficult to generalise due to the limited number of species and environments researched (Rolim et al. 2015; Tricarico et al. 2016; Griesemer et al. 2018; Ramírez-Albores et al. 2019). However, inappropriate responses of individuals to invasive predators can strongly affect native populations (Mooney and Cleland 2001). In amphibians, predation can account for a significant proportion of the total mortality of all their developmental stages (Nyström

et al. 1997; Chivers et al. 2001; Laurila et al. 2002; Gunzburger and Travis 2005). The ability to detect, recognise, and respond to potential predators is, therefore, an important part of antipredatory behaviour (Bennett et al. 2013; Polo-Cavia and Gomez-Mestre 2014), and native populations can have especially serious problems facing the presence of new alien predators (Polo-Cavia et al. 2010; Gomez-Mestre and Díaz-Paniagua 2011; Nunes et al. 2019). In general, embryonic and early larval stages are the most vulnerable to predation (Laurila et al. 2002; Wells 2007), and the ability to respond to the presence of a predator can therefore significantly increase the fitness of an individual and thus the viability of the entire population (Warkentin 1995; Vonesh and Bolker 2005).

Whether intentionally or unintentionally introduced, the recent wide occurrence of the red-eared slider (*Trachemys scripta elegans*) in Europe (GISD 2021) presents a new opportunity to investigate the responses of naive native amphibian populations to a new predator. Although the red-eared slider (hereafter referred to as slider) is not reproductively successful throughout Europe (Cadi et al. 2004; Ficetola et al. 2009; Mikátová and Šandera 2015; Standfuss et al. 2016), even the mere presence of adults may pose a certain risk to native species. In previous studies, we found that the presence of the sliders affects several life history parameters of common frog (*Rana temporaria*) tadpoles, such as movement activity, trajectory of movement (Berec et al. 2016), time to metamorphosis, or size at metamorphosis (Vodrážková et al. 2020). Although sliders are usually still hibernating at the time of common frog breeding (Gibbons et al. 1990; Speybroeck et al. 2016), which eliminates the risk of direct predation, chemical cues (kairomones) released by sliders into the aquatic environment provide amphibians with information about their presence. Since the slider is an opportunistic predator and can consume frog eggs (Ernst and Lovich 2009), some response of common frog embryos is to be expected.

For frog embryos, there are two basic strategies for avoiding predation or significantly reducing its effects: the development of egg unpalatability and hatching plasticity (Wells 2007). The unpalatability of eggs is a passive strategy in which the embryo relies on the predator's inability or unwillingness to consume eggs, which imposes costs on its host even if the host never comes in contact with the predator; environmentally cued hatching is characterised by an embryo's active capability to alter the time of hatching according to the conditions it encounters during embryonic development. Hatching plasticity has been documented many times in amphibian embryos, and predator presence has been shown to trigger early hatching from eggs incubated in both air and water (Chivers et al. 2001; Warkentin 2011). In terrestrially laid eggs, hatching can be stimulated by vibrational cues during the direct physical attacks of predators, such as snakes (Warkentin 1995; Jung et al. 2019), frogs (Vonesh and Bolker 2005), katydids (Poo and Bickford 2014), wasps (Warkentin 2000), or egg-eating fly larvae (Vonesh and Bolker 2005). In aquatic environments, these responses are induced mainly by chemical cues from predators (kairomones) or by chemical cues that are released from injured prey during predation events (Petránka et al. 1987; Dodson 1988; Tollrian 1994; Nicieza 1999, 2000; Laurila et al. 2002; Smith and Fortune 2009).

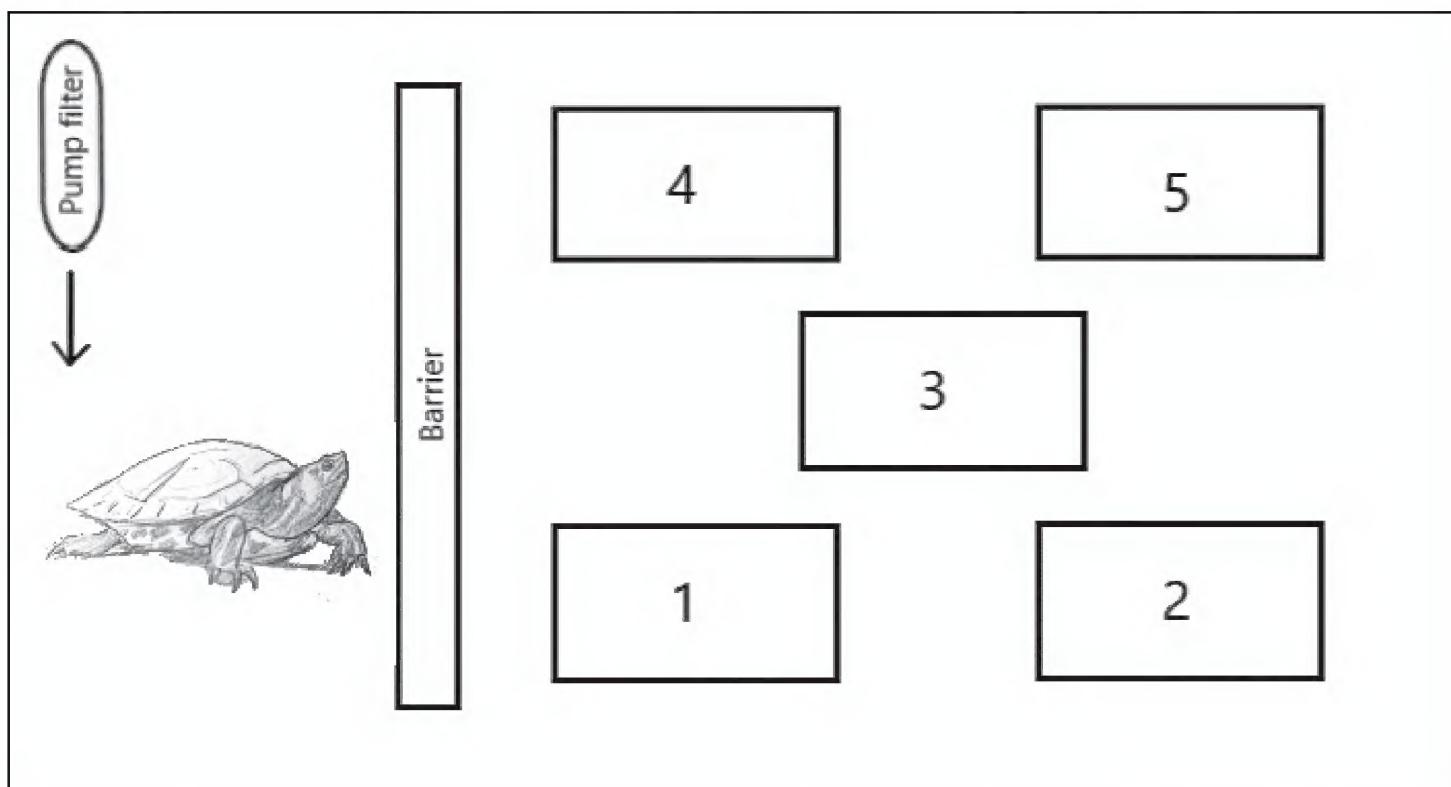
This study aimed to shift our previous focus (Berec et al. 2016; Vodrážková et al. 2020, 2022) to a different developmental stage, namely, embryos in eggs. We investigated whether the presence of a slider can alter the hatching time of common frog embryos. We hypothesised that the presence of a slider would accelerate the hatching time, so the ontogenetic stage and body size at hatching were also measured. The uniqueness of this study lies in the use of a stage-nonspecific predator, which is virtually absent in the literature. At the same time, it is an alien predator from a taxonomic group to which the prey has no common history.

## Materials and methods

Five freshly laid clutches of common frogs were collected in a pool between Holubov and Vrábče, South Bohemia, the Czech Republic ( $48.9078633^{\circ}\text{N}$ ,  $14.3485608^{\circ}\text{E}$ ), on 2 April 2021. Collection locality was monitored daily to collect egg clutches laid during the night before. Neither the slider nor any other species of turtle occurs at the collection locality, so the eggs and their parents are naive prey relative to the turtles. The experiment was performed in six glass tanks – three replications with the sliders and three replications of control without the sliders. Glass tanks (size:  $100 \times 55 \times 50$  cm) filled with 20 cm of aged tap water were equipped with a Claro 300 filter pump ( $300\text{ L.h}^{-1}$ ) and rinsed three times a week. The room temperature was set at  $15^{\circ}\text{C}$  and the datalogger (Dostman LOG200 PDF) recorded a mean air temperature of  $14.8 \pm 0.4^{\circ}\text{C}$  ( $\pm$  S.D.; measured at hourly intervals) during the experiment. Fluorescent tubes ( $2 \times 36\text{ W}$ ) with a light regime of 12 h/12 h were used. During the dark phase of the day, the glass tanks were illuminated with red light to allow permanent monitoring of egg hatching.

Three adult sliders (carapace length: 18 cm, 20 cm, and 21 cm) were used as predators. A slider was placed in each of three glass tanks over the course of three days to release kairomones into the water before the experiment was initiated and fed three times a week with ReptoMin Tetra turtle gammarus. To prevent physical but not chemical contact between the slider and frog eggs, a glass barrier was placed inside each glass tank with a 6 cm gap at both ends so that water could flow freely throughout the tank. On the other side of this barrier, five perforated opaque boxes ( $20 \times 14$  cm) with holes 1 mm in diameter were glued to the bottom of the glass tanks to contain the eggs (Fig. 1).

Six fragments of approximately 150 eggs each were taken from the collected clutch and randomly placed in five boxes, one in each glass tank. This procedure was repeated for all five clutches, so that there were five boxes in each tank with a fragment from each clutch. Each glass tank was continuously monitored using a camera (Niceboy Stream Pro) to distinguish hatched tadpoles occasionally returned to the inside of the egg capsules from tadpoles just before hatching. Hatched tadpoles were counted every 24 h. Hatching was defined as the moment at which the whole hatchling had left the protective jelly of the eggs. To maintain a good processing of the camera recordings



**Figure 1.** Diagram of the glass tank showing the position of the slider (if present) and the boxes for clutch fragments. These were placed randomly in the boxes in each glass tank (see Materials and methods). Three replications with the sliders and three replications without them (control) were used. Slider drawing by Jakub Berec.

(the large number of hatched tadpoles in a small box makes it difficult to count them), hatched tadpoles were transferred every six hours to a depot tank. At the time when half of the eggs in each box had hatched, two tadpoles were taken from the group of tadpoles hatched in the last six hours. These tadpoles were photographed under a stereomicroscope (Olympus SZX 7) and measured (to the nearest 0.01 mm) using QuickPHOTO MICRO 3.2 software. Their developmental phase was determined according to Gosner (1960).

Three different dependent variables connected with different aspects of the hatching of common frog tadpoles with/without the presence of a slider were measured as described above: hatching time, developmental stage, and size at hatching. The potential impact of four independent variables – (1) slider: presence/absence, (2) glass tank: three tanks with slider and three controls without slider, (3) box: five boxes in each glass tank at the same position within the glass tank, and (4) clutch: six fragments of each clutch – on each of the dependent variables was then analysed. Thus, three separate analyses were performed to fulfil the aim of the present study. According to the experimental design, a linear main effect ANOVA model from the general linear model family (GLM) was used for analysis of experimental data (Quinn and Keough 2002). The factor ‘slider’ was set in all three analyses as a fixed factor, as both levels of this factor (presence/absence) were of direct interest to our study and are not interdependent. The other three factors (glass tank, clutch, and box) were set as random factors (Allen 2017). The overall fit of all parameters in the GLM was tested using a test of the sum of squares of whole model versus sum of squares of residuals. An overparameterized

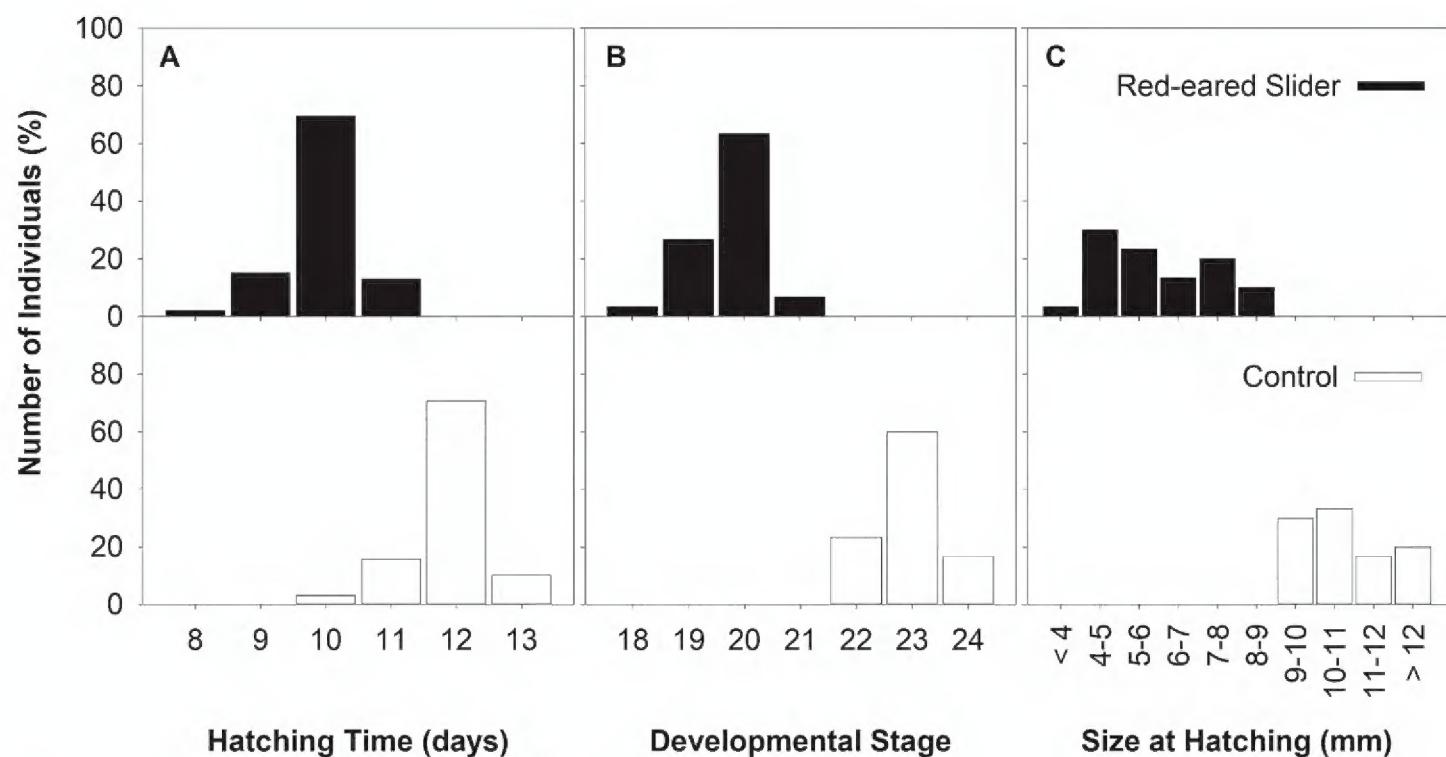
model based on the indicator variable approach (Midway 2019) was used in type III sums of squares test (TIBCO 2017) to represent the effects of all four independent variables (factors) on the dependent variable. Adjusted R-squared was used in the overall fit of all parameters as a measure of the variability explained by the GLM because more than one independent variable was used in the model. The effect sizes of all four partial factors were evaluated using partial eta-squared (Richardson 2011). Given the number of eggs, the statistical significance was assessed at the 99.9% level (Steel et al. 2013). All calculations were performed in Tibco Statistica (TIBCO 2017).

## Results

The GLM for hatching time with the effects of all factors analysed (overall fit of all parameters) was statistically significant (all effect:  $F_{11,2988} = 809.2$ ,  $p < 0.001$ ; adjusted  $R^2 = 0.75$ ). In the partial effects analysis, we found a significant difference in hatching time between the presence and absence of the slider ( $F_{1,2988} = 8672.4$ ;  $p < 0.001$ ). The effect of this factor on hatching time (partial eta-squared 0.74) prevailed over the effect of the other factors. In the absence of the slider, the embryos hatched in  $12 \pm 0.6$  days (mean  $\pm$  S.D.). The presence of the slider accelerated hatching by two days ( $10 \pm 0.6$  days) (Fig. 2A). The partial effects of the random factors were also significant: glass tank ( $F_{2,2988} = 11.7$ ;  $p < 0.001$ ), box ( $F_{4,2988} = 7.6$ ;  $p < 0.001$ ), and clutch ( $F_{4,2988} = 44.1$ ;  $p < 0.001$ ). However, compared to the effect of slider presence, the effect sizes of these factors were negligible (partial eta-squared for clutch: 0.06, glass tank: 0.01, and box: 0.01) (Suppl. material 3: Table S1).

The GLM for developmental stage of all the factors analysed (overall fit of all parameters) was statistically significant (all effect:  $F_{11,48} = 33.2$ ,  $p < 0.001$ ; adjusted  $R^2 = 0.86$ ). In the analyses of partial effects, the presence of the slider was the only significant factor in the model ( $F_{1,48} = 358.0$ ;  $p < 0.01$ ). In the presence of a slider, embryos hatched at developmental stage  $20 \pm 1.5$  (mean  $\pm$  S.D.), while in control, freshly hatched embryos had developed to stage  $23 \pm 1.0$  (Fig. 2B). The developmental stage was not significantly influenced by glass tank, clutch, and box (Suppl. material 3: Table S2).

Similarly to the previous life history parameters, the GLM for size at hatching of all factors analysed (overall fit of all parameters) was statistically significant (all effect:  $F_{11,48} = 23.8$ ,  $p < 0.001$ ; adjusted  $R^2 = 0.81$ ). In the analyses of partial effects, the significant difference was found between the size of freshly hatched embryos in the presence of the slider and without it ( $F_{1,48} = 245.3$ ;  $p < 0.001$ ). In the presence of a slider, the embryos hatched with an average size of  $5.92 \pm 1.460$  mm (mean  $\pm$  S.D.), while in the control, the average size of the freshly hatched embryos was  $10.77 \pm 1.042$  mm (Fig. 2C). As for developmental stage, the presence of the slider was the only significant factor in the model (Suppl. material 3: Table S3).



**Figure 2.** Histogram of **A** hatching time **B** developmental (Gosner) stage, and **C** size at hatching of the embryos of common frogs in the presence of red-eared slider and control.

## Discussion

Developmental plasticity is an adaptive response of anuran embryos and larvae to the risk of predation (Altig and McDiarmid 1999; Benard 2004; Warkentin 2011). Here, we present evidence for the developmental plasticity of common frog embryos in the presence of a red-eared slider and, in addition to a previous study (Vodrážková et al. 2020), provide a comprehensive insight into the influence of this alien predator on the early phases of the common frog life cycle. We have previously shown (Vodrážková et al. 2020) that, in the slider presence, tadpoles of common frogs are capable to modify the duration of larval development. In the present study, we confirmed a similar response in common frog embryos, which hatched earlier in the presence of a slider. At the same time, the embryos were smaller and less developed when exposed to the chemical signals of a predator.

In the presence of stage-specific predators, amphibians can modify the duration of the relevant developmental stage (Chivers et al. 2001; Ireland et al. 2007; Mitchell et al. 2017). In anuran embryos, specifically, the presence of egg predators has mostly been shown to induce early hatching of embryos (Warkentin 1995, 2000; Chivers et al. 2001; Laurila et al. 2001; Johnson et al. 2003; Segev et al. 2015), while tadpole predators induce delayed hatching (Sih and Moore 1993; Laurila et al. 2002; Schalk et al. 2002; Mitchell et al. 2017), thus increasing their chance of survival by escaping possible attacks. However, the slider is not a stage-specific predator, as it is capable of consuming both amphibian eggs and larvae (Brown et al. 1995; Chen 2006; Ernst and Lovich 2009); thus, the allocation of risk between developmental stages of the frog may be more complex in this case (Warkentin 2011). Studies that examine predator effects on the developmental rates of both eggs and larvae are rare because few predators consume both eggs and larvae simultaneously. Muraro et al. (2021) used

a stage-nonspecific predator (*Procambarus clarkii*) and found, in concordance with our results, a reduction in hatching time in *Rana latastei* embryos. However, they did not study larval development. Ireland et al. (2007) solved the problem of predator stage specificity by simultaneously exposing frog eggs to stage-specific predators of eggs (leech: *Nephelopsis obscura*) and larvae (dragonfly: *Aeshna canadensis* nymphs), which resulted in no change in hatching time, while tests with separately acting predators produced the expected response of a reduction in hatching time in the egg predator treatment and an increase in hatching time in the larval predator treatment. This study on embryos and a previous study on tadpoles (Vodrážková et al. 2020) jointly clarify that the embryos/tadpoles of the common frog responded to the presence of a predator by shortening the stage of development during which the embryo/tadpole would be exposed to the predator. It would be interesting to analyse how common frog tadpoles react to the presence of a slider if the entire development from eggs to metamorphosis was taking place with this predator present.

However, some studies have shown that frog embryos, including the common frog, do not always respond specifically to stage-specific predators by shortening hatching time (Laurila et al. 2001, 2002; Schalk et al. 2002; Saglio and Mandrillon 2006; Touchon et al. 2006; Capellán and Nicieza 2010; Touchon and Wojdak 2014). The published differences in embryo responses may correspond to different signal intensities of the presence of a specific predator, and thus the responses to indirect waterborne cues might be weaker than those to the direct, mechanical cues of a predator attack (Warkentin 2011). An evident response to water-borne cues of sliders may be related to a markedly stronger signal of a much larger-sized predator in our experiment compared to commonly tested invertebrate predators. The ability to scale predator danger and adjust hatching time accordingly has been found, for example, in embryos of southern leopard frogs (*Lithobates sphenocephalus*) (Johnson et al. 2003). Moreover, a possible absence of a change in hatching time does not necessarily imply a complete lack of response to the presence of a predator. It may be manifested by other types of response, such as changes in the body shape of tadpoles (Laurila et al. 2001; Saglio and Mandrillon 2006; Mandrillon and Saglio 2007; Touchon and Wojdak 2014) or their behaviour (Saglio and Mandrillon 2006; Touchon and Wojdak 2014).

Native and naive prey can fail to detect the novel predator adequately as a dangerous threat, resulting in no (Cox and Lima 2006; Sih et al. 2010) or inefficient antipredator responses to counter the predator's attack strategies (Strauss et al. 2006; Sih et al. 2010). However, when responses in hatching time in naive prey are detected, they are often explained by the presence of syntopic, taxonomically related predators (Sih et al. 2010; Melotto et al. 2021; Muraro et al. 2021), although the time since invasion may also play an important role (Gómez-Mestre and Díaz-Paniagua 2011; Nunes et al. 2013). Our results suggested that a common evolutionary history is not necessary for a detectable response. Such a result has already been published for tadpole development time (Stav et al. 2007; Vodrážková et al. 2020), but as far as we know, it has not yet been published for the hatching time in frog embryos. An explanation for embryo response to an alien slider may be in the ability of embryos to detect a kind of general "smell of fear" that is elicited by most predators, regardless of taxonomic classification (Sih et al. 2010).

Finding a general tendency in the phenotypic plasticity responses of prey across a broad range of animal predators (different taxa and feeding spectra), environmental and experimental conditions is a challenge even in anurans themselves (Relyea et al. 2018). However, in frogs, the earlier hatching time was generally associated with smaller size at hatching (Chivers et al. 2001; Laurila et al. 2002; Capellán and Nicieza 2007; Ireland et al. 2007) and lower developmental stage (Chivers et al. 2001; Laurila et al. 2002; Capellán and Nicieza 2007; Ireland et al. 2007; Muraro et al. 2021), and our results confirm this relationship. In some cases, earlier hatched tadpoles performed higher growth rate and reached the size of later hatched tadpoles at metamorphosis (Capellán and Nicieza 2007). However, if tadpoles are unable to compensate for their smaller size at hatching, this can impose significant costs in later developmental phases. These costs have been demonstrated through increased mortality during the larval stage (Smith 1987; Warkentin 1995; but see Vonesh and Bolker (2005) where early hatchlings survived better), reduced size at metamorphosis (Vonesh and Bolker 2005; Vodrážková et al. 2020), lower post-metamorphic survival (Berven 1990; Altwegg and Reyer 2003), change of behaviour (Buckley et al. 2005; Capellán and Nicieza 2007), delayed maturity (Smith 1987) and lower reproductive success (Smith 1987).

The hatching time was also influenced by the clutch, glass tank, and box. The clutch effect can be explained by a possible difference in the age of the collected clutches. Although freshly laid clutches were always collected in the morning after the actual reproductive event, differences of several hours in the age of the clutches cannot be excluded. The box effect could be attributed to the different distances of each box from the pump filter and/or the slider compartment. We can rule out a temperature gradient in the experimental room as the most likely cause of the glass tank effect, as regular temperature measurements during the experiment did not detect one. Nevertheless, all partial eta-squared of clutch, glass tank, and box are an order of magnitude lower than partial eta-squared for slider presence/absence. This confirms the importance of the slider presence/absence on the hatching time. Moreover, the statistical significance of the above-mentioned random factors need not be functionally relevant.

Although the results are fairly straightforward, we are aware of certain limitations of our experiment. First, the five clutches used originated from a single pool. For this reason, the general validity of our results cannot be confirmed, as some studies also point to a genetic component of variability in some features of ontogenetic development (Lind et al. 2008; to our knowledge, the genetic component of hatching time variability in frogs has not yet been investigated). An additional potential statistical complication could be the placement of multiple boxes in a glass tank and multiple eggs (a fragment of clutch) in a box. Having individual eggs in individual tanks with their own maintenance system and with a separate water supply from the glass tank with or without a slider would prevent this issue. At the same time, it solves the problem of different box distances from the pump filter and/or the slider compartment. However, although this solution is technically feasible, division of the clutches into individual eggs remains problematic in terms of embryo survival.

Our work added a slider as an additional predator inducing changes in the embryonic developmental rate in Ranidae. Since the impact of earlier embryo hatching (lower body

size and lower stage of development) on fitness has been confirmed in several frog species (Warkentin 1995; Laurila et al. 2002; Vonesh and Bolker 2005; Touchon et al. 2013), the same impact can be expected for the common frog. The existence of defensive responses in slider-exposed embryos may reduce the threat that the spreading of this invasive species poses in Europe. On the other hand, the reduced size at hatching and developmental stage of common frog hatchlings represents additional risks of negative fitness impacts, and at the very least, the presence of sliders in non-native areas should receive increased attention.

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All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Czech Ministry of Agriculture, Department of Animal Welfare according to article No. 15, section 2 of the act registered under number 9103/2009-17210.

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## Supplementary material I

### Hatching time

Authors: Magda Vodrážková, Irena Šetlíková, Josef Navrátil, Michal Berec

Data type: excel file.

Explanation note: Data of hatching time (days) with four independent variables – slider presence (0/1), glass tank (1–6), box (1–5), and clutch (1–5) ( $n = 3000$ ).

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## Supplementary material 2

### Developmental stage, size

Authors: Magda Vodrážková, Irena Šetlíková, Josef Navrátil, Michal Berec

Data type: excel file.

Explanation note: Data of developmental (Gosner) stage and size at hatching (mm) with four independent variables – slider presence (0/1), glass tank (1–6), box (1–5), and clutch (1–5) ( $n = 30$ ).

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## Supplementary material 3

### Tables 1–3

Authors: Magda Vodrážková, Irena Šetlíková, Josef Navrátil, Michal Berec

Data type: Docx file.

Explanation note: Results of linear main effect ANOVA models for hatching time (Table S1), developmental stage (Table S2), and size at hatching (Table S3). All three tables include the overall fit of all parameters including adjusted R<sup>2</sup> and then univariate results for all factors tested including partial eta-squared. Abbreviations: SS = Sum of Squares, d.f. = Degrees of Freedom, and MS = Mean Square.

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